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# Segmental expression of *Hoxa-2* in the hindbrain is directly regulated by *Krox-20*

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## SUMMARY

**The hindbrain is a segmented structure divided into repeating metameric units termed rhombomeres (r). The *Hox* family, vertebrate homologs of the *Drosophila HOM-C* homeotic selector genes, are expressed in rhombomere-restricted patterns and are believed to participate in regulating segmental identities. *Krox-20*, a zinc finger gene, has a highly conserved pattern of expression in r3 and r5 and is functionally required for their maintenance in mouse embryos. *Krox-20* has been shown to directly regulate the *Hoxb-2* gene and we wanted to determine if it was involved in regulating multiple *Hox* genes as a part of its functional role. *Hoxa-2* is the only known paralog of *Hoxb-2*, and we examined the patterns of expression of the mouse *Hoxa-2* gene with particular focus on r3 and r5 in wild type and *Krox-20*$^{-/-}$ mutant embryos. There was a clear loss of expression in r3, which indicated that *Hoxa-2* was downstream of *Krox-20*. Using transgenic analysis with *E. coli lacZ* reporter genes we have identified and mapped an r3/r5 enhancer in the 5′ flanking region of the *Hoxa-2* gene. Deletion analysis narrowed this region to an 809 bp *Bgl*II fragment, and in vitro binding and competition assays with bacterially expressed Krox-20 protein identified two sites within the enhancer. Mutation of these Krox-20 sites in the regulatory region specifically abolished r3/r5 activity, but did not affect neural crest and mesodermal components. This indicated that the two *Krox-20* sites are required in vivo for enhancer function. Furthermore, ectopic expression of *Krox-20* in r4 was able to transactivate the *Hoxa-2/lacZ* reporter in this rhombomere. Together our findings suggest that *Krox-20* directly participates in the transcriptional regulation of *Hoxa-2* during hindbrain segmentation, and is responsible for the upregulation of the r3 and r5 domains of expression of both vertebrate group 2 *Hox* paralogs. Therefore, the segmental phenotypes in the *Krox-20* mutants are likely to reflect the role of *Krox-20* in directly regulating multiple *Hox* genes.**

Key words: *Krox-20*, *Hoxa-2*, hindbrain segmentation, rhombomeres, transcriptional regulation, transgenic mice, enhancers

## INTRODUCTION

Segmentation is an important mechanism in regionalisation of the hindbrain which has been highly conserved during vertebrate evolution (Lumsden, 1990; Wilkinson, 1993; Keynes and Krumlauf, 1994). In early vertebrate embryos a similar number of periodic swellings transiently appear in the developing hindbrain where they are termed rhombomeres (r). Cellular analysis in chicken embryos has demonstrated that rhombomeres are lineage-restricted compartments (Fraser et al., 1990; Birgbauer and Fraser, 1994), with reduced mixing and cell-cell communication between adjacent segments (Guthrie and Lumsden, 1991; Martinez et al., 1992; Guthrie et al., 1993). There is a tight correlation between specific rhombomeric segments and the organisation of branchiomotor nerves, sensory ganglia, neuronal development, branchial arches and generation/migration of cranial neural crest (Lumsden and Keynes, 1989; Lumsden et al., 1991; Serbedzija et al., 1992; Sechrist et al., 1993; Birgbauer et al., 1995). On the basis of axonal organisation of the branchiomotor and sensory nerves (Lumsden and Keynes, 1989) and that alternate rhombomeres have similar properties with respect to cell mixing and neural crest generation and migration (Guthrie and Lumsden, 1991; Graham et al., 1993, 1994; Guthrie et al., 1993; Sechrist et al., 1993), there appears to be an underlying two-segment periodicity to the general organisation of the hindbrain. Together this cellular data strongly argues that rhombomeric segments are fundamental units involved in generating regional diversity in the CNS and in head morphogenesis.

Underlying this morphological organisation, at the molecular level expression studies have revealed that transcription factors, growth factors and receptor tyrosine kinases display rhombomere-restricted patterns of expression (Wilkinson et al., 1988, 1989a, b; Gilardi-Hebenstreit et al., 1992; Becker et al., 1994; reviewed by Wilkinson, 1993). In vertebrates, many members of the *Hox* gene family have anterior limits of expression in the hindbrain which map precisely to rhombomere boundaries, and in addition there are high levels of expression in specific rhombomeres (Murphy et al., 1989; Wilkinson et al., 1989b; Sundin and Eichele, 1990; Hunt et al., 1991; Prince and Lumsden, 1994; reviewed by McGinnis and Krumlauf, 1992; Keynes and Krumlauf, 1994). Furthermore these restricted patterns of *Hox* expression arise before the morphological appearance of rhombomeres, suggesting that *Hox* genes play a role in regulating rhombomeric processes. Loss-of-function mutations in the mouse *Hoxa-1* gene, generated by targeted disruption, severely affect hindbrain patterning, and in particular, formation of r5 (Carpenter et al., 1993; Dolle et al., 1993; Mark et al., 1993). However, no overt rhombomeric phenotypes have been observed in other targeted *Hox* mutations (reviewed by Krumlauf, 1994), which could be due to functional compensation or synergistic interactions between different *Hox* genes expressed in the same region (Condie and Capecchi, 1994). In gain-of-function experiments ectopic expression of the *Hoxa-1* gene results in the transformation of r2 to an r4 identity (Zhang et al., 1994), demonstrating that in a manner analogous to their *Drosophila HOM-C* counterparts, the *Hox* genes can regulate segmental identity.

Very little is known about how the rhombomere-restricted patterns of *Hox* expression are established in the hindbrain. *Krox-20* encodes a zinc finger transcription factor (Chavrier et al., 1988, 1990) and is expressed in the presumptive r3 and r5 domains of many vertebrate embryos (Wilkinson et al., 1989a; Nieto et al., 1991; Bradley et al., 1992; Oxtoby and Jowett, 1993), and loss-of-function mutations in the mouse *Krox-20* gene severely affect these rhombomeres (Schneider-Maunoury et al., 1993; Swiatek and Gridley, 1993). In the case where the *Krox-20* gene has been disrupted by insertion of the *E. coli lacZ* gene, it is possible to follow *Krox-20* expressing cells in the mutants. In these embryos we have observed that r3 and r5 are initially formed, but fail to develop properly and are rapidly eliminated (Schneider-Maunoury et al., 1993). Hence *Krox-20* is required for the maintenance of r3 and r5. *Krox-20* has been shown to be directly involved in the regulation of the *Hoxb-2* gene in r3 and r5 (Sham et al., 1993), which links the *Krox-20* mutant phenotype with the regulation of *Hox* genes. However, it is unclear whether the segmental abnormalities in the mutant mice arise from the influences on *Hoxb-2* alone or involve other members of the *Hox* family.

The four vertebrate *Hox* clusters arose by duplication and divergence from a common ancestor, and as a consequence there are highly related genes in each of the complexes which represent paralogous groups (McGinnis and Krumlauf, 1992). Not only are paralogous genes similar in structure, but in the hindbrain they have similar rhombomere-restricted patterns of expression (Hunt et al., 1991; Keynes and Krumlauf, 1994), suggesting a real potential for functional compensation or redundancy. The only paralog of *Hoxb-2* is *Hoxa-2*, and these genes have different anterior boundaries of expression in the neural tube in both chicken and mouse embryos where *Hoxa-2* is present in r2 (Krumlauf, 1993; Prince and Lumsden, 1994; Frasch et al., 1995). Despite these differences it has been shown in the mouse that *Hoxa-2* is also expressed at higher levels in r3 and r5 (Hunt et al., 1991; Krumlauf, 1993). Therefore, *Krox-20* might also be involved in regulating this gene, although loss-of-function mutations in the *Hoxa-2* gene do not display abnormalities in r3 and r5 (Gendron-Maguire et al., 1993; Rijli et al., 1993).

In this study we have examined the expression of *Hoxa-2* in homozygous *Krox-20* mutants and found that it is altered, suggesting that it is downstream of *Krox-20*. Furthermore, we have used a combination of transgenic and in vitro biochemical analysis to identify an r3/r5 enhancer in the *Hoxa-2* locus, and show that this enhancer has Krox-20 binding sites necessary for in vivo activity. The results demonstrate that *Hoxa-2* is also a direct target of *Krox-20* during hindbrain segmentation, indicating that *Krox-20* is involved in the control of multiple members of the *Hox* family.

## MATERIALS AND METHODS

### DNA constructs and transgenic analysis

The *Hin*dIII-*Not*I fragment from a cosmid containing the *Hoxa-2* and *Hoxa-3* genes was subcloned and mapped. Fragments for transgenic analysis were inserted into the *Sma*I site of the *lacZ* reporter vector pBGZ40 (Yee and Rigby, 1993; Studer et al., 1994). The respective constructs contained the following genomic fragments: #1, 4.0 kb *Eco*RI; #2 2.6 kb *Eco*RI-*Bgl*II partial digest; #3 1.9 kb *Eco*RI-*Bgl*II; #4 3.1 kb *Acc*I-*Eco*RI; #5 1.2 kb *Bgl*II-*Eco*RI; #6 0.8 kb *Bgl*II. Construct #7 contained the specific mutant *Krox-20* sites. In some cases fragments were also tested using a minimal *Hoxb-4/lacZ* reporter vector (construct #8 in Whiting et al., 1991). Transgenic mice were generated by microinjection of fertilised eggs from crosses between $F_1$ hybrids (CBA×C57), identified by PCR analysis and embryos assayed for β-galactosidase activity as perviously described (Whiting et al., 1991; Sham et al., 1993; Marshall et al., 1994; Studer et al., 1994). For ectopic expression constructs the *Krox-20* cDNA was inserted into a 7.5 kb *Eco*RV fragment containing the *Hoxb-1* gene and r4 enhancer (Marshall et al., 1994; Studer et al., 1994; Popperl et al., 1995). The *Krox-20* cDNA was inserted into the 5′ untranslated region following removal of sequences from –45 bp upstream to 101 bp downstream of the *Hoxb-1* ATG. A line with construct #2 was used to make double transgenic mice in the *Krox-20* transactivation experiments. All sequencing was performed by the dideoxy method on both strands.

### Whole-mount in situ hybridisation and combined β-galactosidase staining

Whole-mount in situ hybridisation was done on mouse embryos as described by Wilkinson and Green (1990); Becker et al. (1994) using a 300 bp *Apa*I-*Pst*I fragment from the 3′ untranslated region of *Hoxa-2* cloned into pGem4Z to generate a T7 transcribed riboprobe labelled with digoxigenin. β-galactosidase staining on embryos used for in situ hybridisation was performed as previously described (Schneider-Maunoury et al., 1993), except that staining reactions were terminated earlier to prevent masking the in situ signal. The embryo genotype for the *Krox-20* mutants was determined by PCR on yolk sac DNA according to Schneider-Maunoury et al. (1993).

### Electrophoretic mobility shift assay (EMSA)

EMSA and competition assays were performed as described previously with control or Krox-20 containing bacterial extracts (Chavrier

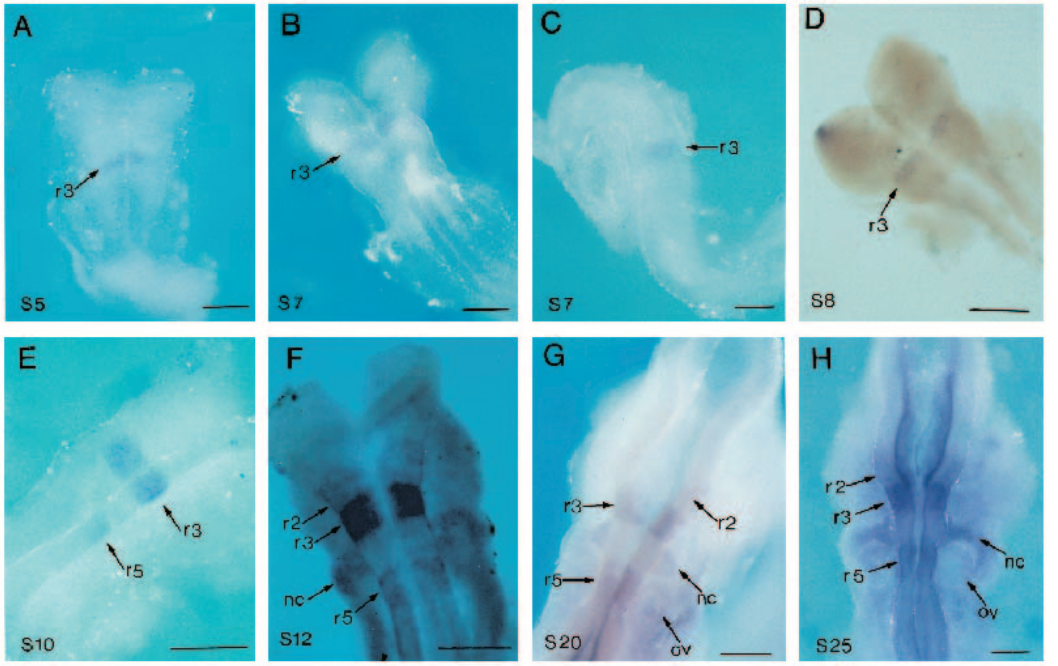


**Fig. 1.** Time course of *Hoxa-2* expression in the developing hindbrain. Dorsal (A,B) and lateral (C) views of 5- (A) and 7-somite (B,C) embryos showing appearance of *Hoxa-2* in the presumptive r3 domain. (D) Flat mount of an 8-somite embryo showing the rostrocaudal restriction of the expression in the r3. (E) 10-somite embryo demonstrating activation of *Hoxa-2* in r5 and persistence in r3. (F) A dorsal view of a 12-somite embryo showing strong expression in r3 and lower levels in r2, r5 and in the neural crest adjacent to r4. (G,H) Expression in 20- and 25-somite embryos respectively, demonstrating a homogenous low level of *Hoxa-2* signal in the neural tube posterior to the r1/r2 boundary, upon which higher levels in r3 and r5 are superimposed. Rhombomeres are indicated by arrows. The number of somites (s) of the embryo is indicated on each panel. nc, neural crest cells; ov, otic vesicle. Scale bar, 200 μm for A-C, G and H, and 100 μm for D and F.

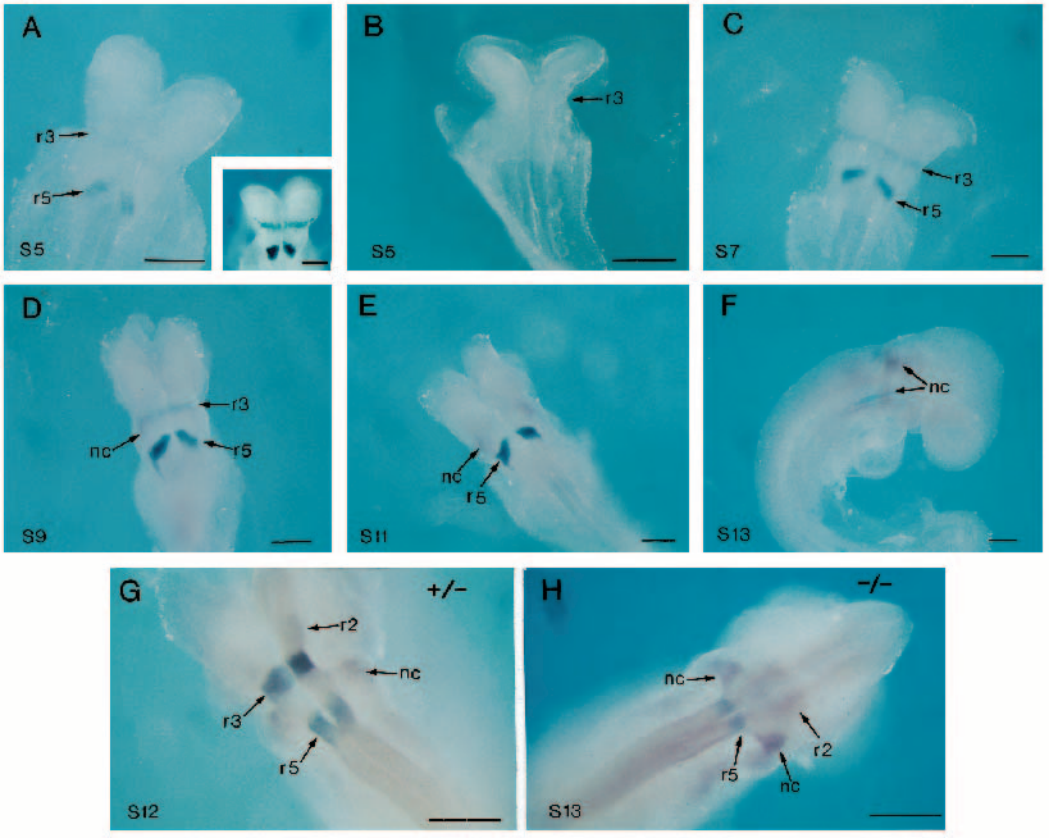


**Fig. 2.** Effect of the *Krox-20* mutation on *Hoxa-2* expression in the hindbrain. (A,C-E) Homozygous *Krox-20* mutant embryos doubly labelled for *Krox-20* by β-galactosidase activity and for *Hoxa-2* by in situ hybridisation, demonstrating the absence of *Hoxa-2* in r3. The insert in A shows a 5-somite embryo stained for a longer time to show more clearly the presence of *Krox-20*-expressing cells in r3. (B) Dorsal view of a 5-somite *Krox-20*$^{-/-}$ embryo labelled only with the *Hoxa-2* probe showing the absence of transcripts in r3. (F) Lateral view of a 13-somite *Krox-20*$^{-/-}$ embryo labelled only with the *Hoxa-2* probe, showing expression in neural crest (nc) cells migrating into the second branchial arch. Note also the presence of *Hoxa-2*-positive neural crest cells in D-E. (G,H) Double labelling of 12-somite heterozygous (G) and 13-somite homozygous (H) *Krox-20* mutant embryos showing colocalisation of *Krox-20* and *Hoxa-2* in r3 and r5 of the heterozygous embryo and only in r5 of the homozygous mutant embryo, where r3 has already disappeared. Note the presence of *Hoxa-2* transcripts in r2, neural crest and posterior neural tube in both embryos. Rhombomeres are indicated by arrows. The number of somites (s) of the embryo is indicated in each panel. nc, neural crest cells. Scale bar, 200 μm.

et al., 1990; Nardelli et al., 1991). Bacterially expressed protein extracts were prepared essentially according to Kadonaga et al. (1987), with the slight modifications described previously (Sham et al., 1993). The probes were purified by electrophoresis on 6% polyacrylamide gels, after hybridisation of the two strands. They were labelled at their 3′ termini with $^{32}$P using the Klenow enzyme. 5.0 ng of the 257 bp *Hin*dIII fragment or 0.5 ng of the Cons oligonucleotide were used in each assay. Competitions were performed with unlabelled purified double-stranded oligonucleotides whose relative concentrations were determined by ethidium bromide staining after electrophoresis on non-denaturing polyacrylamide gels. The sequence of the oligonucleotides (one strand only) were:

Cons 5′-GCGGGGGCG-3′; Mut 5′-GCGGCGGCG-3′;

site 1: 5′-TTTTCACCCACGCAGCCT-3′; site 2: 5′-AATGCTGTGGGCAGCCCT-3′

1+2 5′-TTTTCACCCACGCAGCCTGACAAAGCCCAATGCGTGGGCAGCCCT-3′

1*+2* 5′-TTTTCACCGACGCAGCCTGACAAAGCCCAATGCTGTCGGCAGCCCT-3′

## RESULTS

### Hoxa-2 expression in wild-type and *Krox-20* mutant embryos

As a first step in determining if *Hoxa-2* is subject to regulation by *Krox-20* we wanted to examine *Hoxa-2* expression in r3 and r5 in *Krox-20* mutants. Since the homozygous *Krox-20* null mutation leads to a progressive disappearance of r3 and r5 during hindbrain development (Schneider-Maunoury et al., 1993; Swiatek and Gridley, 1993), the analysis of its effect on gene expression in these rhombomeres is complicated and requires the existence of a window of time during which both the rhombomeres are still present in the mutant and the gene expressed. This is necessary to eliminate the trivial possibility that loss of expression is simply due to the absence of r3 and r5. Therefore, we first had to examine in greater detail the time course of *Hoxa-2* expression in these segments in the wildtype hindbrain.

Low levels of *Hoxa-2* expression are first observed around 7.5 dpc, as previously reported by Frasch et al. (1995). Upregulation of *Hoxa-2* in the region of presumptive r3 is detected around the 5-somite stage (Fig. 1A), where its expression continues to increase up to the 15-somite stage (Fig. 1B-F). At the 20- to 25-somite stage expression in r3 begins to decrease (Fig. 1G,H). Upregulation of *Hoxa-2* expression in r5 is first detected at the 10-somite stage, and in r5 the levels of expression are generally lower than those in r3. As previously shown, the gene is also expressed at a low level in r2 and r4 from the 12-somite stage and in neural crest emigrating from r4 (Fig. 1 and Hunt et al., 1991; Krumlauf, 1993; Frasch et al., 1995).

In homozygous *Krox-20* null embryos, we have followed the presence of r3 and r5 cells by the expression of the hybrid *Krox-20/lacZ* gene resulting from the insertion of the *lacZ* sequence into the *Krox-20* locus (Schneider-Maunoury et al., 1993). The fusion gene expression faithfully recapitulates that of the endogenous *Krox-20* gene (Schneider-Maunoury et al., 1993; Topilko et al., 1994). *Krox-20/lacZ*-expressing cells corresponding to the prospective r3 are observed between the 0- and 10-somite stages (Fig. 2A,C,D and data not shown). In situ hybridisation with a *Hoxa-2* probe on a 5-somite stage *Krox-20*$^{-/-}$ mutant embryo shows that there is no upregulation of *Hoxa-2* in prospective r3 even though cells corresponding to future r3 are present (Fig. 2B). This does not represent a delay in upregulation of *Hoxa-2* in r3, because we have performed double labelling on embryos at various stages to reveal both β-galactosidase activity and *Hoxa-2* mRNA (Fig. 2A,C-H). Presumptive r3 is lost in homozygous *Krox-20*$^{-/-}$ mutant embryos, as determined by β-gal staining, between the 9- and 11-somite stages (Fig. 2D,E), and no upregulation in r3 was ever detected during this period. Despite these effects on r3, apparently normal *Hoxa-2* expression was observed in r2, r4 and r4 neural crest throughout later stages (Fig. 2E-H). Therefore, *Hoxa-2* is not upregulated in r3 before it is lost in the *Krox-20*$^{-/-}$ embryos.

Similar analysis of *Hoxa-2* in r5 is more difficult in *Krox-20*$^{-/-}$ mutants because the level of upregulation in r5 is low in wild-type embryos (Fig. 1E-H) during the period when presumptive r5 cells are still present in the *Krox-20*$^{-/-}$ mutants (Fig. 2F,H). We have never observed the low level upregulation of *Hoxa-2* in r5 in the *Krox-20*$^{-/-}$ embryos at the 12- to 13-somite stages. Highest upregulation of *Hoxa-2* in r5 occurs around the 20- to 25-somite stage in wild-type embryos (Fig. 1H) and we would have liked to examine expression in the mutants at this stage to be sure that upregulation in r5 had not occurred. However in the mutants β-gal staining cells representing presumptive r5 are missing at this stage, precluding analysis (data not shown). In conclusion, our results indicate that *Hoxa-2* is under the control of *Krox-20* in r3, but with respect to regulation in r5 the data are inconclusive.

### Mapping of a *Hoxa-2* r3/r5 enhancer

These observed changes in the *Hoxa-2* expression pattern raise the possibility that *Krox-20* could be directly involved in aspects of *Hoxa-2* regulation, in a manner analogous to that previously shown for its paralogous gene *Hoxb-2* (Sham et al., 1993). To test this possibility we have used transgenic mice to identify the *cis*-regulatory regions required for upregulation of *Hoxa-2* expression in r3 and r5. Fig. 3 maps the intergenic domain between *Hoxa-2* and *Hoxa-3* in the mouse where we have identified an r3/r5 control region, and summarises the regulatory regions examined and their activity in transgenic analysis. A 4.0 kb *Eco*RI fragment upstream of the *Hoxa-2* ATG initiation codon was inserted into a vector containing the *E. coli* β-galactosidase *(lacZ)* reporter gene under control of a minimal promoter (Construct #1, Fig. 3). The only expression of the transgene in the neural tube was detected in the hindbrain in r3 and r5 (Fig. 4A,B). While there was no expression in r4 itself, the neural crest (nc) cells migrating from r4 into the second branchial arch showed strong staining. Posterior of the otic vesicle and lateral to the neural tube both neural crest and paraxial mesoderm expressed the transgene.

This fragment has been shown to function on several promoters (data not shown), suggesting that there is an r3/r5 enhancer located in the 5′ flanking region and we used deletion analysis to map its position. Constructs #2 and #4 generally had patterns of expression in r3 and r5 and the neural crest identical to those containing the entire 4.0 kb *Eco*RI fragment (Fig. 4C,E). The 2.7 kb *Eco*RI-*Bgl*II fragment in construct #2 worked in both orientations supporting the idea that the regulatory element has the properties of an enhancer. Construct #4 produced a few embryos (2 of 13) with high levels of staining

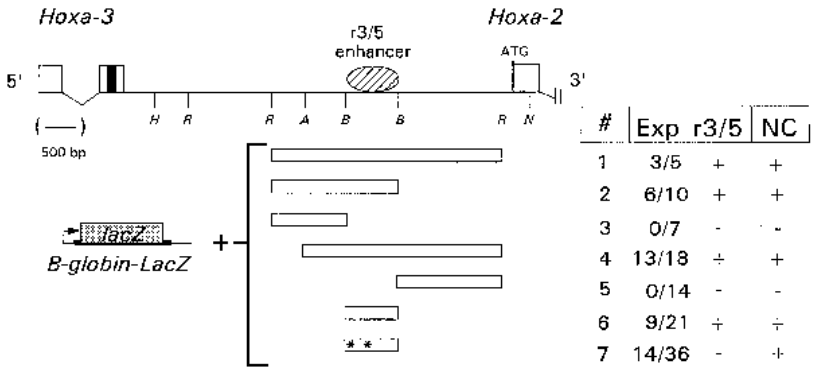


**Fig. 3.** Mapping and transgenic analysis of the *Hoxa-2* r3/r5 enhancer. On the left is a map of the relative positions of the *Hoxa-2* and *Hoxa-3* genomic loci, with the respective restriction fragments used for transgenic constructs indicated below. *β-globin-lacZ* refers to the pBGZ40 reporter vector used to test regulatory activity in the transgenic analysis. On the right is a table indicating construct number (#), the patterns of expression in r3, r5 and neural crest (NC), and the fraction of the embryos positive for either pattern (Exp). In each construct all of the embryos that expressed the transgene had consistent patterns, and the fraction not showing expression is presumably due to integration site effects. In construct #7 point mutations (**) in the Krox-20 binding sites have been introduced by site directed mutagenesis. Restriction sites H, *Hin*dIII, R, *Eco*RI, A, *Acc*I, B, *Bgl*II, N, *Not*I.

in r3 and r5, but only a few positive cells in the r4 neural crest population (Fig. 4F). This suggests that r3/r5 expression is independently regulated from that of the neural crest and has been differentially affected by the integration site in some cases. The only expression from the *lacZ* reporter in embryos containing constructs #3 and #5 occurred in ectopic locations (Fig. 4D,G). The minimal overlap between constructs #2-5, suggested that the r3/r5 activity of the enhancer might map to an 809 bp *Bgl*II fragment, hence we tested its functional activity. Indeed, the *Bgl*II fragment alone (construct #6) was sufficient to direct reporter expression in r3 and r5 and the neural crest cells (Fig. 4H).

## Analysis of expression mediated by the r3/r5 enhancer

To investigate expression in more detail, four transgenic lines containing the r3/r5 enhancer were generated and displayed identical staining patterns. Fig. 4I-K shows a time course of expression from 8.25-10.5 days post coitum (dpc) for one of the lines. The transgene is initially expressed in r3 at about 8.0 dpc, and a day later high levels of expression are seen in both r3 and r5. Flat mounts and coronal sections show that the expression in the hindbrain is restricted to rhombomeres 3 and 5 (Fig. 5A). Expression then is downregulated in r3 around 10.0 dpc (Figs 4K, 5D), and subsequently in r5 at later stages. The overall temporal progression of appearance and downregulation of expression from the *Hoxa-2* transgene mirrors that of *Krox-20* (Wilkinson et al., 1989a; Sham et al., 1993), except that it is slightly delayed. This is consistent with the idea that *Krox-20* may be directly involved in regulating the *Hoxa-2* gene.

Outside the hindbrain, sections reveal that the reporter is expressed in the trigeminal and facial cranial sensory ganglia and mesenchymal cells of the second branchial arch (Fig. 5). In the trigeminal ganglion only a small number of positive cells are observed (Fig. 5E), presumably originating from r3 neural crest, which contributes to a subset of first arch derivatives (Sechrist et al., 1993; Nieto et al., 1995). Sections through posterior regions show that staining occurs in sclerotome and myotome derivatives, lateral mesoderm, and dorsal root ganglia (Fig. 5F). Another site of endogenous *Krox-20* expression is the boundary cap cells which surround the exit points of the motor nerves (Wilkinson et al., 1989a; Topilko et al., 1994), and the *Hoxa-2/lacZ* reporter gene is also expressed in these boundary cap cells (Fig. 5C). Hence the enhancer mediates reporter expression which overlaps with endogenous domains of *Krox-20* in a number of locations.

## The 809 bp *Bgl*II r3/r5 enhancer contains *Krox-20* binding sites

On the basis of the boundary cap and r3/r5 restricted expression and similar temporal regulation of the transgene, we investigated the possibility that the *Bgl*II fragment might contain Krox-20 binding sites. The fragment was subjected to digestion with *Hin*dIII and *Ava*II, producing four subfragments (Fig. 6A), which were analysed by electrophoretic mobility shift assays (EMSA) using bacterial extract containing the Krox-20 protein. Only the 257 bp *Hin*dIII fragment gave rise to retarded complexes (Fig. 6B and data not shown). This suggests that the other fragments do not contain a Krox-20 binding site. Incubation of the 257 bp fragment with increasing concentrations of the Krox-20 protein lead to the formation of two major complexes, C1 and C2 (Fig. 6B). At high concentrations of the extracts, most of the DNA was incorporated into the complex of lowest mobility (C2). Both complexes were competed with an excess of an oligonucleotide carrying a high affinity consensus binding site for Krox-20 (Cons) (Nardelli et al., 1991). No competition was observed with a related oligonucleotide (Mut), having an identical sequence, except for a point mutation which reduces the affinity for Krox-20 (Nardelli et al., 1992) demonstrating the specificity of Krox-20 binding. In conclusion, this analysis suggests that the 809 bp enhancer contains at least two Krox-20 binding sites, both of which are located within the 257 bp *Hin*dIII fragment.

## Characterisation of the Krox-20 binding sites

Fig. 6A shows the sequence of the 809 bp *Bgl*II fragment and the sequence of the 257 bp *Hin*dIII fragment in particular was searched for the presence of motifs similar to the high affinity Krox-20 binding site consensus, 5′ GCGNGGGCG 3′. Two such motifs were identified (Fig. 6C), the first of which (5′ GCGTGGGTG 3′; site #1) is present in reverse orientation. It is very close to the consensus, differing only on the 8th position, which is known to be degenerate because this base is not directly contacted by the protein (Chavrier et al., 1990; Paveletich and Pabo, 1991). The second motif,

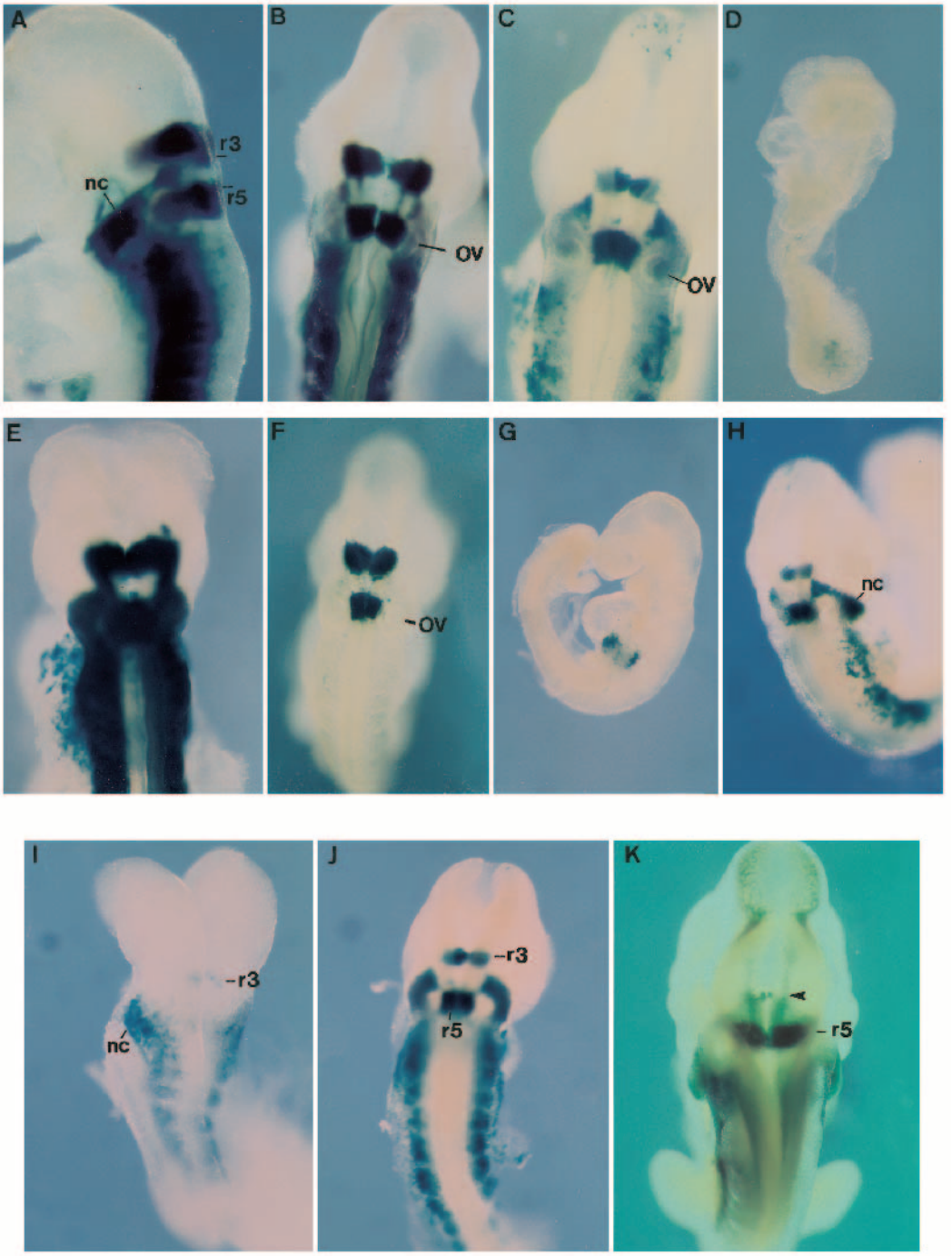


**Fig. 4.** Deletion analysis of the *Hoxa-2* r3/r5 enhancer and temporal expression in transgenic embryos. (A-H) Transgene expression in 9.5 dpc embryos containing constructs: #1 (A,B); #2 (C); #3 (D); #4 (E,F); #5 (G) and #6 (H) according to Figure 3. A-C, E and H show identical patterns of expression with high levels in r3 and r5, r4 neural crest (nc), and both paraxial mesoderm and neural crest posterior to the r5. F shows an embryo which has high levels of staining in the r3 and r5, but with a very small number of expressing cells in the r4 neural crest. Constructs #3 (D) and #5 (G) mediate only ectopic expression in the tail, limb bud and forebrain suggesting that essential elements of the enhancer are located within the 809 bp *Bgl*II fragment (H). (I-K) Time-course of reporter expression in line transgenic for constructs #2. Four independent lines all had identical patterns of expression. Dorsal views of (I) 8.25 dpc, (J) 9.5 dpc and (K) 10.5 dpc embryos showing that the transgene is first expressed in r3, then appears in both r3 and r5 and is subsequently downregulated first in r3 in a pattern that mirrors that of *Krox-20*. nc, neural crest cells; ov, otic vesicle.

5′ CTGTGGGCA 3′ (site #2), is more distant from the consensus and was not expected to constitute a high affinity binding site. Both motifs were tested for their capacity to bind Krox-20 in EMSA. Oligonucleotides carrying these motifs were used as competitors against the high affinity Cons oligonucleotide (Fig. 7A). As predicted by the sequence, we found that oligonucleotide 1 carrying the first motif competed almost as efficiently as the Cons oligonucleotide itself and corresponds to a high affinity Krox-20 binding site. In contrast, motif 2 was poorly effective in the competition assay (Fig. 7A). Competitions performed with higher concentrations of oligonucleotide 2 as well as direct binding assays, indicated nevertheless that it bound Krox-20, requiring about ten-fold higher concentrations than the Cons oligonucleotide (data not shown). We also synthesized another oligonucleotide carrying both sites, because sites 1 and 2 are separated by only 20 bp in their natural configuration (Figs 6A, 7B). This combined oligonucleotide was found to be a better competitor that site 1 alone at the same concentrations (Fig. 7A). This suggests that Krox-20 binding to sites 1 and 2 is cooperative and that, in the presence of site 1, site 2 can behave as a high affinity binding site. To establish definitively that the Krox-20 binding activity of the oligonucleotide containing sites 1+2 was only due to the two

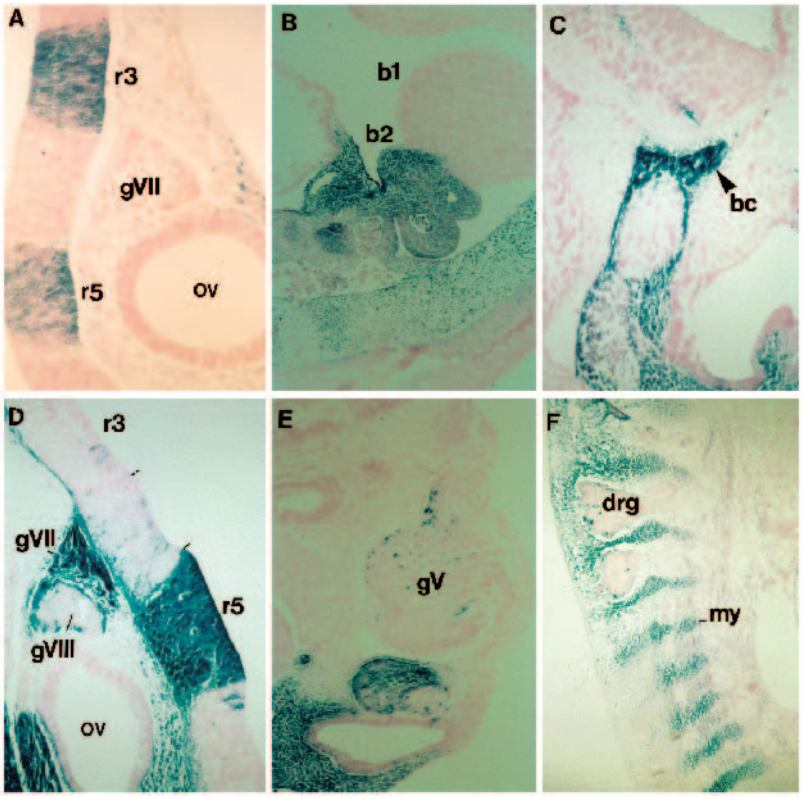


**Fig. 5.** Analysis of *Hoxa-2/lacZ* expression in transgenic lines. (A) Coronal section thorough the hindbrain of a 9.0 dpc embryo showing restricted expression in r3 and r5. A small number of positively stained cells in the developing facial ganglion are present in lateral regions. (B) Sagittal section of a 11.5 dpc embryo with restricted expression up to the boundary between the first and second branchial arches. Axial mesenchyme is also stained. (C) Sagittal section of an 11.5 dpc embryo showing staining in the boundary cap (bc) cells surrounding the exit point of the facial nerve. (D) Coronal section of a 12.5 dpc embryo showing that expression is downregulated in r3, strong in r5, the facial ganglion (gVII), and lateral neural crest derivatives. (E) Sagittal section of a 12.5 dpc transgenic embryo showing staining in a few cells of the trigeminal ganglion (gV), and extensive staining in the facial ganglion and mesenchymal neural crest cells migrating into the branchial arches. (F) Sagittal section of a 12.5 dpc embryo showing staining in the dermomyotomes (my), sclerotomes and dorsal root ganglia (drg). OV, otic vesicle.

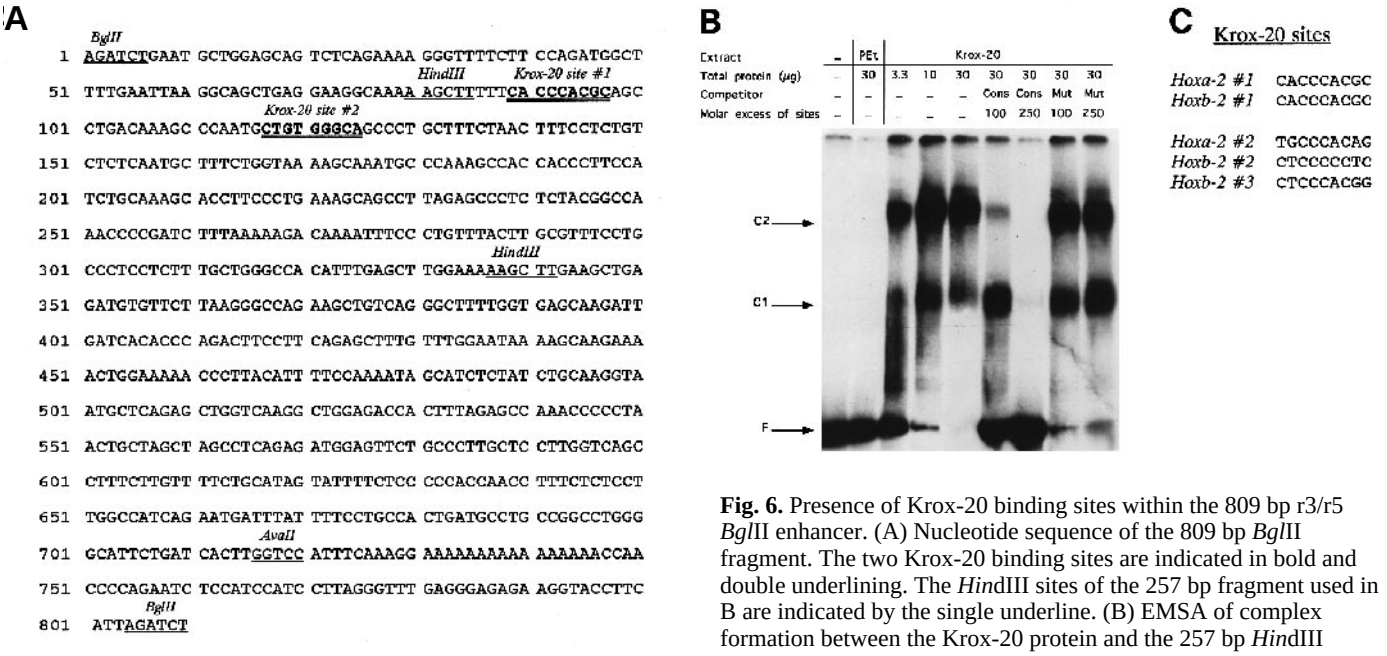


**Fig. 6.** Presence of Krox-20 binding sites within the 809 bp r3/r5 *Bgl*II enhancer. (A) Nucleotide sequence of the 809 bp *Bgl*II fragment. The two Krox-20 binding sites are indicated in bold and double underlining. The *Hin*dIII sites of the 257 bp fragment used in B are indicated by the single underline. (B) EMSA of complex formation between the Krox-20 protein and the 257 bp *Hin*dIII fragment. At the top are indicated the amounts of control bacterial extract (PEt) or extract containing Krox-20 and the types of unlabelled oligonucleotides used as competitors in the EMSA. (Cons), consensus high affinity Krox-20 binding site; (Mut), a mutated version of this consensus which does not bind Krox-20. F, free DNA and C1 and C2 the two complexes formed in the presence of Krox-20. (C) Comparison of the high and low affinity *Krox-20* binding site in the *Hoxa-2* enhancer, with those from the *Hoxb-2* enhancer (Sham et al., 1993). Note the considerable divergence in site #2-#3.

Krox-20 sites identified by the sequence comparison we eliminated them. A related oligonucleotide (1*+2*, Fig. 7B) was synthesized carrying in each of the two sites a single G to C point mutation at the central position of the site, which is known to inactivate binding to the consensus site (Nardelli et al., 1992). EMSA analysis indicated that the mutations had

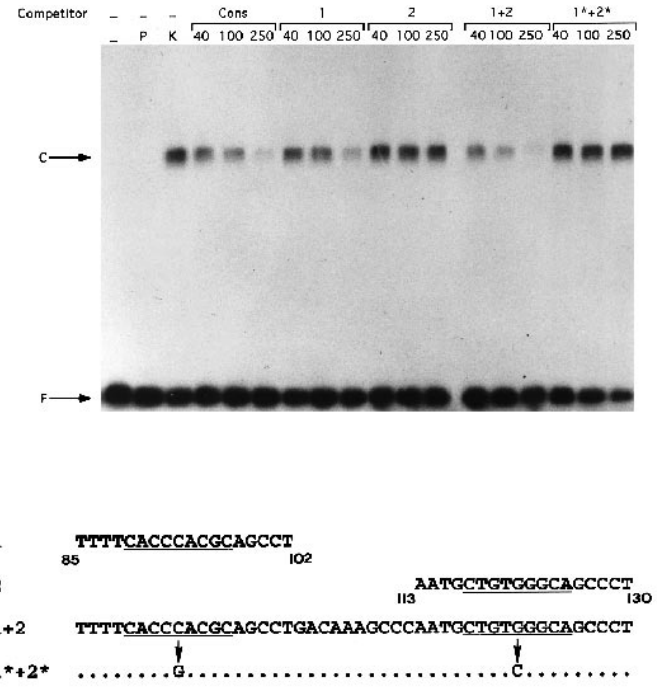


**Fig. 7.** Krox-20 binds co-operatively to two sites on the 257 bp *Hin*dIII fragment. Sequences of the oligonucleotides (bottom) used in the EMSA competition experiments (top), with the *Krox-20* sites underlined. The labelled probe, an oligonucleotide containing a consensus high affinity Krox-20 binding site (Cons), was incubated with 7 μg of total protein of a bacterial extract containing Krox-20. Competitors and molar excess are indicated above. The three first lanes correspond to EMSAs, with the following bacterial extracts: (–) no added extract; (P), control bacterial extract without Krox-20 (7μg); (K), bacterial extract containing Krox-20 (7 μg). F and C indicate free and complexed oligonucleotide, respectively.

completely eliminated the ability of this oligonucleotide ($1^*$+$2^*$) to compete for Krox-20 binding (Fig. 7A).

### The two Krox-20 binding sites are required in vivo for r3/r5 enhancer activity

In order to determine if the two *Krox-20* binding sites defined by the in vitro analysis are involved in the r3/r5 enhancer activity, we introduced the same point mutations tested in vitro ($1^*$+$2^*$) into the context of the 809 bp *Bgl*II fragment (construct #7) for transgenic analysis. Transgene expression in the second arch neural crest and posterior regions was unaffected by the mutations in the *Krox-20* sites (Fig. 8). However, in all the embryos generated using the mutated version, staining in r3 and r5 was specifically abolished. Embryos were analysed at a number of stages between 8.0-10.5 dpc to ensure that this was not merely a failure to maintain r3/r5 expression which had been established earlier. Expression in r3 and r5 was never detected at any stage. These patterns of expression mediated by the construct with *Krox-20* mutant sites indicate that r4 neural crest expression is regulated independently of that in r3 and r5. These results indicate that the two *Krox-20* binding sites are necessary in vivo for the functional activity of the r3/r5 enhancer in the *Bgl*II fragment, suggesting that Krox-20 mediates the upregulation of *Hoxa-2* in r3 and r5 through interaction with these sites.

### Transactivation of *Hoxa-2* by Krox-20

Ectopic expression of *Krox-20* in transgenic mice was used to examine the ability of *Krox-20* to upregulate *Hoxa-2* through the r3/r5 enhancer. Using a control region from the *Hoxb-1* gene, which directs restricted expression of reporter genes in r4 (Marshall et al., 1994; Studer et al., 1994; Popperl et al., 1995) we generated a transgenic construct (*r4/Krox-20*) that ectopically expressed *Krox-20* in this rhombomeric segment. As shown above (Fig. 4I-K) lines carrying the *lacZ* reporter under control of the r3/r5 enhancer, do not express the transgene in r4. However when the *r4/Krox-20* ectopic expression construct was introduced into this transgenic background, reporter expression was specifically induced in r4 (Fig. 9D,E). Furthermore, this analysis was also performed by co-injecting the *r4/Krox-20* construct with the *lacZ* reporter linked to several versions of the r3/r5 enhancer and identical results were obtained (Fig. 9A-C). In the co-injection experiments we eliminated the possibility that the *lacZ* reporter activation in r4, was a *cis*-effect of the r4 enhancer, by using the *Hoxb-1* r4 element without *Krox-20* as a control (data not shown). In addition we have used a neural enhancer from the *Hoxb-4* gene (region A, Whiting et al., 1991) to ectopically express *Krox-20* in the neural tube posterior to r6, and found that it also activates the *Hoxa-2* r3/r5 enhancer (data not shown). Together our findings demonstrate both that the *Krox-20* sites are necessary for enhancer activity and that Krox-20 protein is able to transactivate transgene expression. This argues that the *Hoxa-2* gene is a direct in vivo target of *Krox-20*.

## DISCUSSION

The regional identity of segments in the embryonic hindbrain is thought to be regulated by the combinatorial expression of *Hox* genes, which involves a complex cascade responsible for establishing and maintaining rhombomere-restricted expression. In the present study we have shown that the zinc finger gene *Krox-20* is implicated in regulating *Hoxa-2* expression in r3 and r5. By deletion analysis in transgenic mice we have defined a 809 bp enhancer with two Krox-20 binding sites shown to be essential for the upregulation of *Hoxa-2* in r3 and r5. Furthermore ectopic *Krox-20* expression transactivates expression mediated by this enhancer. Thus, our data suggest that *Hoxa-2* is a direct target for *Krox-20* during the process of hindbrain segmentation.

### *Krox-20* regulates multiple *Hox* genes

We found that in *Krox-20* mutant embryos there is an absence of *Hoxa-2* upregulation in r3, and the loss of expression in r5 is less obvious. In addition, the identification of an r3/r5 enhancer in the 5′ flanking region of the gene, which contains *Krox-20* binding sites required for its activity, argues that normal upregulation of *Hoxa-2* in both r3 and r5 is dependent upon *Krox-20*. It might be possible that other regulatory elements are involved in regulating r3 and r5 expression of *Hoxa-2*. However, in our analysis the enhancer described in this paper is the only regulatory region between *Hoxa-2* and *Hoxa-3* capable of mediating r3/r5 expression. Furthermore, Frasch et al. (1995) scanned a 16 kb genomic region encompassing *Hoxa-2* and *Hoxa-1* and were unable to find regulatory components involved in mediating the upregulation in r3 and

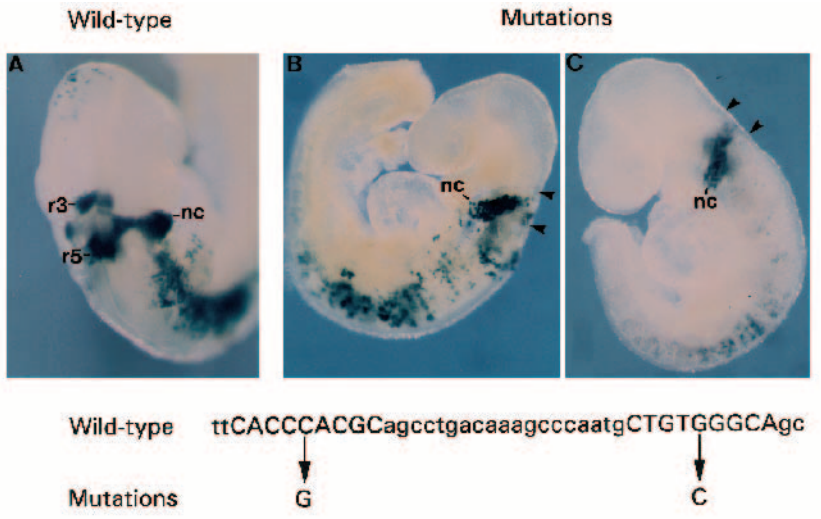


**Fig. 8.** Krox-20 binding sites are required for r3/r5 enhancer activity in vivo. (A) Expression in a 9.5 dpc embryo with the wild-type 809 bp r3/r5 enhancer (construct #6). (B,C) Lateral views of reporter expression in two independent transgenic embryos carrying single point mutations in each of the Krox-20 binding sites within the 809 bp enhancer (construct #7). Note that r3 and r5 expression is specifically abolished, and expression in r4 neural crest (nc) and posterior regions is unaltered indicating the Krox-20 sites defined in vitro are necessary for r3/5 enhancer activity only. Below the panels are the sequences of the wild-type and mutant constructs in the region spanning the two Krox-20 binding sites. The arrows indicate the nucleotide changes, which were identical to those used in the EMSA experiments in Fig. 7.

r5, despite the fact that they reconstructed most of the other domains of *Hoxa-2* expression. Therefore, in the *Hoxa* complex, analysis over 22 kb spanning the three genes, *Hoxa-1* to *Hoxa-3*, indicates the 809 bp *Bgl*II fragment is the only r3/r5 enhancer identified, and we conclude that this regulatory region is likely to be the essential control element responsible for upregulation in r3 and r5 of the endogenous *Hoxa-2* gene.

While this data may suggest that *Hoxa-2* is important for r3/r5 patterning, the lack of an obvious rhombomeric phenotype in *Hoxa-2* null mutants indicates that it is not absolutely required (Gendron-Maguire et al., 1993; Rijli et al., 1993). This does not mean that *Hoxa-2* is not involved in regulating r3 and r5 properties, because there could be functional compensation by another *Hox* gene. In fact our previous analysis on the regulation and expression of its paralog, *Hoxb-2*, in normal and *Krox-20* mutant embryos revealed that in r3 and r5 *Hoxb-2* is also directly controlled by *Krox-20* (Schneider-Maunoury et al., 1993; Sham et al., 1993). Therefore for normal hindbrain patterning, *Krox-20* appears to have a direct role in regulating multiple *Hox* genes in r3 and r5.

## Properties of the *Hoxa-2* r3/r5 enhancer

The expression of *lacZ* reporter genes mediated by the *Hoxa-2* and *Hoxb-2* enhancers show similar patterns. In addition to the restricted expression in r3 and r5 there are overlaps in a number of domains outside the hindbrain. In particular *Hoxa-2* is also expressed in boundary cap cells, which are non-neuronal support cells marking the exit points of the motor nerves. Since *Krox-20* mutants also display a phenotype in Schwann cells (Topilko et al., 1994), it is possible that it has a role in regulating *Hoxa-2* and *Hoxb-2* in locations other than r3 and r5.

Based on our initial analysis the *Krox-20* sites of *Hoxa-2* and *Hoxb-2* alone are not sufficient for r3/r5 regulation. The evolutionary relationship between *Hoxa-2* and *Hoxb-2* and the fact that they are regulated by *Krox-20* would indicate that cooperation with similar or identical factors might be required for the activity of both enhancers. We had hoped to define binding sites for such cooperating factors by using

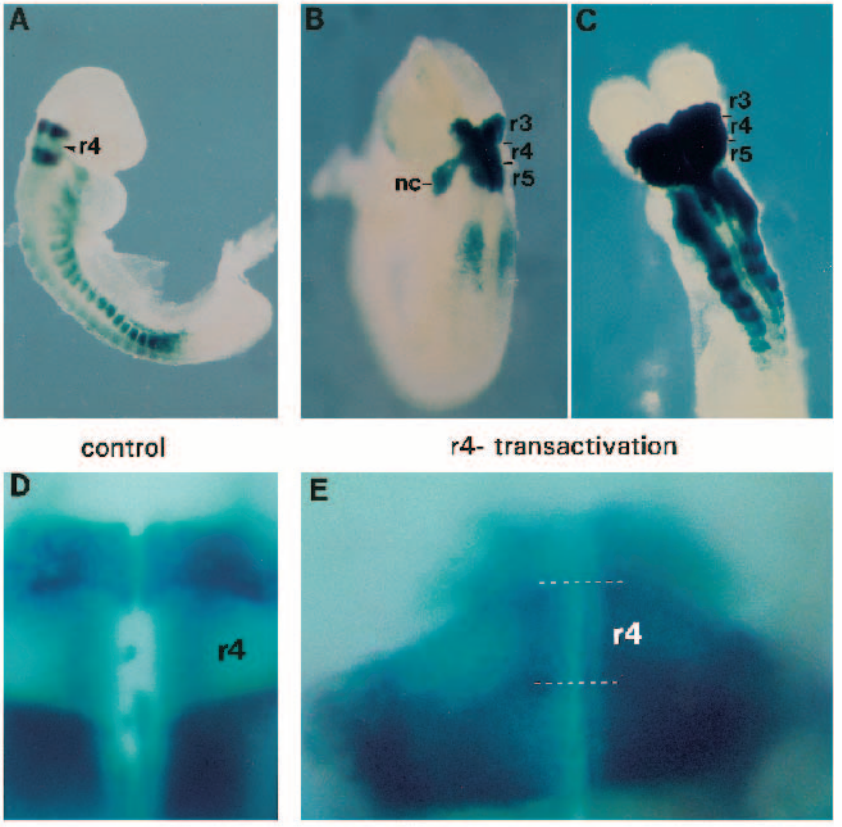


**Fig. 9.** Ectopic expression of *Krox-20* transactivates the r3/r5 enhancer. (A,D) Control embryos from the transgenic line carrying construct #2, showing reporter expression throughout r3 and r5, but not r4. (E) In this transgenic reporter line, the ectopic expression of the Krox-20 protein in r4 specifically induces staining in this rhombomere. (B,C) β-galactosidase expression mediated by various r3/r5 enhancer constructs is also induced specifically in r4 upon ectopic *Krox-20* expression in co-injection experiments. nc, neural crest.

sequence comparison to identify conserved blocks that might be required, but this has not been informative. In both the *Hoxa-2* and *Hoxb-2* enhancers one high affinity Krox-20 binding site has been completely conserved (Fig. 6C, sites #1), but in general there is poor overall conservation of the enhancer nucleotide sequence, including the other Krox-20 binding sites (Fig. 6C). If the sites for common factors are small or moderately degenerate or if different factors are required for *Krox-20* interactions in *Hoxa-2* and *Hoxb-2* regulatory regions, then it may be more important to examine the homologs in other species, which has proved useful for analysis of *Hoxb-1* and *Hoxb-4* (Marshall et al., 1994; Studer et al., 1994; Aparicio et al., 1995; Morrison et al., 1995; Popperl et al., 1995).

### Regulatory conservation between paralogous *Hox* genes

Because *Hoxa-2* and *Hoxb-2* arose through duplication and divergence from a common ancestor it would not be surprising that they shared common regulatory mechanisms if these arose before the duplication events. However, there are considerable differences in the expression of these two paralogs. *Hoxa-2* is strongly expressed in r2 and at low levels in r4, while *Hoxb-2* is not expressed in r2 and has high levels in r4 (Krumlauf, 1993). Furthermore, the enhancer mediating r4 expression of *Hoxb-2* is located in the 5′ flanking region of the locus and directs expression throughout the rhombomere and its associated neural crest (Sham et al., 1993), while the *Hoxa-2* r4 enhancer is positioned within the first intron and mediates only dorsal expression (Frasch et al., 1995). These differences and the lack of conserved sequences in the r4 enhancers, suggests that they reflect independent regulatory mechanisms. Therefore, there has not been a general or global conservation of segmental regulation between these paralogs. Hence, the common role of *Krox-20* in regulating r3/5 expression is a unique highly conserved aspect that presumably reflects a fundamental feature in the regulation of the vertebrate ancestral *Hox* complex.

### *Hoxa-2* regulation in r4 and r4 neural crest

A primary phenotype of *Hoxa-2* null mutants was found to be centred in the neural crest derivatives of the second branchial arch, where there was an anterior homeotic transformation of mesenchymal structures to a first arch identity (Gendron-Maguire et al., 1993; Rijli et al., 1993). This occurred in the absence of any detectable changes to r4, indicating that the neural crest had not adopted an anterior fate due to a change in the identity of the rhombomere from which it was derived. In our analysis, also present in the r3/r5 enhancer were elements capable of imposing expression in neural crest derived from r4, but not in r4 itself. The neural crest regulatory region functioned independently of the components in the enhancer required for *Krox-20*-dependent expression in r3 and r5. Recently, it has been shown that *Hoxa-2* has a separate r4 regulatory element in the intron, which directs expression in a dorsal subset of r4 but not in second arch crest (Frasch et al., 1995). This implies that second arch neural crest expression of *Hoxa-2* is regulated independently from hindbrain segmentation. In agreement with this, in chick embryos transposed rhombomeres display independent expression in neural tube and neural crest (Prince and Lumsden, 1994). Therefore, the neural crest phenotypes in the *Hoxa-2* mutants may reflect a primary role for *Hoxa-2* in directing morphogenetic events (mediated by the r4 neural crest enhancer we identified above) in response to signals in the environment of the second branchial arch, rather than a secondary defect from changes in the hindbrain *Hox* code.

### Are *Hox* genes the sole or primarily targets of *Krox-20* in the hindbrain?

The rhombomeric phenotypes in *Krox-20* mutants could arise solely as a result of the altered regulation of *Hoxa-2* and or *Hoxb-2* alone. In r3 these are the only members of the *Hox* family to be expressed. However, members of paralogous group three are also expressed in r5, and in particular *Hoxb-3* and *Hoxa-3* display a specific upregulation in r5 (Hunt et al., 1991; Keynes and Krumlauf, 1994). Hence, *Krox-20* could be involved in regulating even more *Hox* genes in r5 which contribute to the mutant phenotypes, and it will be important to investigate the mechanism for upregulation of *Hoxa-3* and *Hoxb-3* in r5.

In addition to *Hox* genes there are an increasing number of other transcription factors, growth factors and receptor tyrosine kinases which show restricted expression in the hindbrain (Wilkinson et al., 1988, 1989a; Gilardi-Hebenstreit et al., 1992; Becker et al., 1994; reviewed by Wilkinson, 1993). Preliminary evidence in *Krox-20* mutants indicates that the *Eph* family member *Sek-1*, which is expressed in r3 and r5 (Nieto et al., 1992) is also downstream of *Krox-20* in the regulatory cascade (TS and PC, unpublished data). It will therefore be important to determine whether other genes in the hindbrain are controlled directly or indirectly by *Krox-20*, and in the latter case whether this regulatory link is mediated by *Hox* genes.

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